PAIN

Pain 93 (2001) 101–106

www.elsevier.nl/locate/pain

Research papers

Mapping a gene for neuropathic pain-related behavior following peripheral neurectomy in the mouse

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Received 26 October 2000; received in revised form 24 January 2001; accepted 30 January 2001

Abstract

Total hindpaw denervation in rodents elicits an abnormal behavior of licking, scratching and self-injury of the anesthetic limb ('autotomy'). Since the same denervation produces phantom limb pain and anesthesia dolorosa in humans, autotomy has been used as a model of human neuropathic pain. Autotomy is an inherited trait in rodents, attributable to a few genes of major effect. Here we used recombinant inbred (RI) mouse lines of the AXB-BXA RI set to map a gene for autotomy. Autotomy levels following unilateral sciatic and saphenous nerve section were scored daily for 36 days, using a standardized scale, in all 23 RI lines available for this set. We used a genetic map of 395 marker loci and a permutation-based statistical method for categorical data to assess the statistical significance of mapping results. We identified a marker on chromosome 15 with statistical support ($P = 0.0003$) in the range considered significant for genome-wide scans in the mouse. Several genes located in this chromosomal region encode for neural functions related to neuropathic pain and may indicate targets for development of novel analgesics.

Keywords: Pain; Autotomy; Genetics; Mouse model; Gene mapping

1. Introduction

Nerve injury in humans from amputations and other causes often produces chronic pain, typified by episodes of spontaneous pain, and dysesthetic and painful sensations evoked by various stimuli. Phantom limb pain occurs in about 80% of amputees (Sherman, 1997), but its intensity, frequency and duration varies widely, even for similar lesions (Jensen and Nikolajsen, 1999). This variation and its modifiability by emotional, cultural, situational, cognitive and other environmental factors, suggest that chronic pain is a complex trait. There are several animal models of chronic pain, primarily for neuropathic pain following partial limb denervation (Zeltser and Seltzer, 1994; Bennett et al., 2000). Autotomy was the first widely adopted, and is the only model for spontaneous human pain syndromes triggered by total limb denervation such as peripheral neurectomy brachial plexus avulsion, dorsal root sections or limb amputation (Wall et al., 1979; Coderre et al., 1986; Zeltser and Seltzer, 1994). Although autotomy is not a normal response by humans to total limb denervation or chronic pain, it occurs in species ranging from rodents to primates. Onset and course of autotomy are closely correlated with the timing and amount of ectopic firing of neural impulses from sensory nerve fibers at the severed nerve end and from the dorsal root ganglia (Devor and Seltzer, 1999). Autotomy responds to drugs known to be effective in treatment of neuropathic pain including local anesthetics, opiates, benzodiazepines, tricyclic antidepressants and sympatholytic agents (Wiesenfeld-Hallin, 1984; Coderre et al., 1986; Seltzer et al., 1989; Devor and Seltzer, 1999). Finally, autotomy is accompanied by an increase in plasma corticosterone levels, an indicator of stress (reviewed by Coderre et al., 1986). These multiple lines of evidence support autotomy as a model of dysesthesia and neuropathic pain (but see Kruger (1992) for an alternative interpretation).

Autotomy levels vary greatly among different inbred, outbred and selected lines of mice and rats raised under identical conditions (Wall et al., 1979; Wiesenfeld and Hallin, 1981; Devor et al., 1982; Defrin et al., 1996; Mogil et al., 1999; Shir et al., 2001), demonstrating that genetic factors play a major role in autotomy in these rodents. Lines of rats have been selected for high versus low autotomy levels (Devor and Raber, 1990), further

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0304-3959/01/$20.00
PII: S0304-3959(01)00295-0

confirming the genetic basis of this trait. Crosses of these lines produce distributions of progeny consistent with autotomy differences due primarily to a single autosomal recessive gene with minor modifiers (Devor and Raber, 1990), but prior to our study no gene has been mapped. Here we used recombinant inbred (RI) mouse lines to carry out a genome scan for autotomy using a gene mapping method previously developed for this breeding design (Diehl and Erickson, 1997).

2. Methods

There are currently over ten different mouse RI sets available, each derived from a different combination of progenitor inbred mouse lines, but only a few sets have a sufficient number of independently-derived lines and a genetic map dense enough to provide sufficient statistical power for linkage analysis. Therefore, we first needed to assess whether any of the available RI sets were suitable for our mapping study. Sets established from a pair of progenitor inbred lines where both lines exhibit high autotomy (or both exhibit low autotomy) would not segregate the major genetic variation among the derived RI lines and thus would not be useful for our aim. The pair of progenitor inbred lines of an ideal RI set for our mapping study would differ substantially in autotomy. A recent study reported autotomy levels of 11 inbred mouse lines (Mogil et al., 1999) and this helped narrow our search, but assessment of additional inbred lines was still necessary. We tested autotomy of male and female mice of A/J, AKR/J, C57L/J and C57BL/6J lines following hindpaw denervation as described below. We then measured the autotomy phenotype in the RI set selected for the analysis and used these data to map a gene for this neuropathic pain-related behavior.

2.1. Animals

This study followed NIH regulations for humane experimentation on animals, and the guidelines of the International Association for the Study of Pain. We used male and female mice produced by The Jackson Laboratory, Bar Harbor, ME, USA, of the A/J, AKR/J, C57L/J and C57BL/6J inbred lines ($n = 10$ mice/group). We also tested males of the AXB-BXA RI lines ($n = 8$–9 mice/line). These lines were established by crossing females of the A/J inbred line with males of the C57BL/6J inbred line (AXB) or females of the C57BL/6J line with males of the A/J line (BXA). Hereafter, A/J is abbreviated as A and C57Bl/6J as B lines, as these animals have been used to construct the AXB-BXA RI set. Following the initial cross, separate lines are established by inbreeding separate lines for over 20 generations until most of the animals have become homozygous and identical within each separate line, with a genome that is a mixture of the genomes of the two parental lines. All animals were age 12 weeks at the time of surgery, weighing 21.2–46.8 g (males) and 17.4–36.4 g (females). All mice were evaluated at the same season of the year, by the same experimenter (Z.S., blinded to the animals' line identity during surgery and scoring of autotomy). All mice were housed in identical environmental conditions. We found no correlation between the following variables and autotomy at the end of the experiment on day 36 postoperatively: hour of the day the denervation was done, weight of the animal on day 0, depth of anesthesia, incidence and time of onset of swelling in the paw, autotomy scores of mice housed in neighboring cages, or RI line of mice in neighboring cages. Throughout the experiment animals were housed singly per cage, at a SPF-grade vivarium (ambient temperature of 22 $\pm$ 0.5°C; day/night cycle of lights on at 07:00 and off at 19:00; water and food supplied ad libitum; mice consumed standard rodent chow (NIH-31 produced by Zeigler Bros. Inc., PA, USA). Unilateral total hindpaw denervation (Wall et al., 1979) was produced under Nembutal anesthesia (50 mg/kg, i.p., supplemented when needed). The sciatic and saphenous nerves on the same side were tightly ligated with 5–0 silk in two locations spaced 2 mm apart and cut between the ligatures, with an equal number of mice were operated on the right and left sides. The proximal ligature was done first, to introduce only one injury discharge to the CNS (Seltzer et al., 1991). The wounds were closed layer by layer, suturing the muscles with 5–0 silk and stapling the skin. The mice were treated with topical and systemic antibiotics. Following recovery from anesthesia, animals were returned to the vivarium. Complete denervation of the hindlimb was verified 7 days postoperatively by the absence of a flexion response to pinching the paw, including the toes. Levels of pain behavior were followed daily for 36 days postoperatively. Scoring was carried out by a single observer and was based on a scale developed by Wall et al. (1979). A score of one was assigned for injury of the nail portion only (without bleeding or soft tissue injury) of two or more toes. An additional point was added for tissue injury (with bleeding) of each half toe, up to a maximum of 11 points if both the distal and proximal halves of all toes exhibited tissue injury. Animals reaching maximal permitted scores of 11 were euthanized promptly and this score used for statistical analyses.

2.2. Statistical analysis

For gene mapping analyses, we adapted a database of 547 markers previously developed for Quantitative Trait Locus (QTL) mapping of AXB-BXA RI lines (Sampson et al., 1998). We removed 89 markers with heterozygous genotypes or with data missing for one or more of the 23 RI lines included in our study, and 56 adjacent markers with identical strain distribution patterns in our 23 RI lines because these would cause problems for interpreting permutation testing results. We also removed seven markers with inconsistent map locations in the AXB-BXA (Sampson et al., 1998) and the Mouse Genome Database (Blake et al.,

2000). These modifications produced a set of 395 markers used in our study and available on request.

As discussed in the text, autotomy scores at day 36 postoperatively were highly bimodal and not continuous, so we used a gene mapping strategy designed for binary, categorical data with the RI genetic design (Diehl and Erickson, 1997). We classified scores of 0 and 1 as indicating no autotomy and scores ≥2 as exhibiting autotomy. The statistical method, described in detail previously (Diehl and Erickson, 1997), uses logistic regression to model the proportion of animals in each RI line 'affected' by autotomy. For each marker, a contrast is then specified to assess differences in frequency of autotomy among those RI lines which inherited the A progenitor allele versus RI lines which inherited the B progenitor allele. This method accounts for the sample size of animals in each RI, and the variation among RI lines sharing the same (A or B) progenitor allele defined separately for each marker. To assess how likely a contrast of the magnitude seen for D15Mit28 is expected to occur by chance when conducting a search of the entire mouse genome, appropriately adjusting for the multiple comparisons, we permuted the autotomy phenotypes associated with each RI line (sampling without replacement) 2000 times and then calculated the contrast statistic for each of the 395 markers for each permutation (790 000 analyses total). The frequency of markers in the permuted data set with contrast χ^2 statistics greater than that observed for D15Mit28 in the real data set was used to estimate the significance of this finding. Analyses were conducted using the GENMOD program of SAS for Windows NT, Version 6.12 (SAS Institute, Cary, North Carolina). All program code and macros for conducting permutation testing are available on request.

3. Results

3.1. Identification of RI lines

Fig. 1A shows that the greatest pairwise difference in autotomy scores was observed between male mice of the A/J and C57BL/6J lines. Females of these two inbred lines also differed, though not quite as much as males (Fig. 1B). The C67BL/6J line exhibited a standard error of only 0.2 and the A/J line had a standard error of 1.6 after postoperative day 12, and so the differences between these lines are highly significant. Since this set has 23 independently-derived lines available and over 800 genetic markers already characterized (Sampson et al., 1998), we therefore decided to assess autotomy in male mice of this RI set for our gene mapping study.

3.2. Mapping a gene for autotomy

Fig. 2 shows the frequency of autotomy scores ≥2 observed in male mice of the 23 RI lines and the progenitor A and B inbred strains. About half of the RI lines show low levels of autotomy comparable to the B progenitor line, while the other half show high levels comparable to the A parent. For this figure and for gene mapping analyses, we classified animals exhibiting scores of 0 and 1 as exhibiting no autotomy because a score of 1 refers to injury of only the nails. Scores ≥2 involve a frank tissue injury to the toes and involving bleeding, so these animals were defined as showing autotomy. We used this binary, categorical variable for gene mapping statistical analyses because the distribution of scores of individual animals within many of the RI lines were bimodal. By day 36, many animals displayed either little or no autotomy (i.e. scores of 0 or 1) or displayed the highest score of 11, and few animals displayed intermediate scores. It wasn't possible to transform this distribution of scores into a continuous, normally distributed variable. In our protocol, therefore, the autotomy differences among RI lines could only be measured by differences in the percentage of animals displaying autotomy or not, rather than by continuous differences in the level of the trait expressed. In fact, for individual RI lines this percentage is highly correlated with the mean autotomy score for the line (data not shown), the variable that was presented for the progenitor inbred lines in Fig. 1.

For gene mapping analyses, we used a previously established database of genetic markers designed for QTL

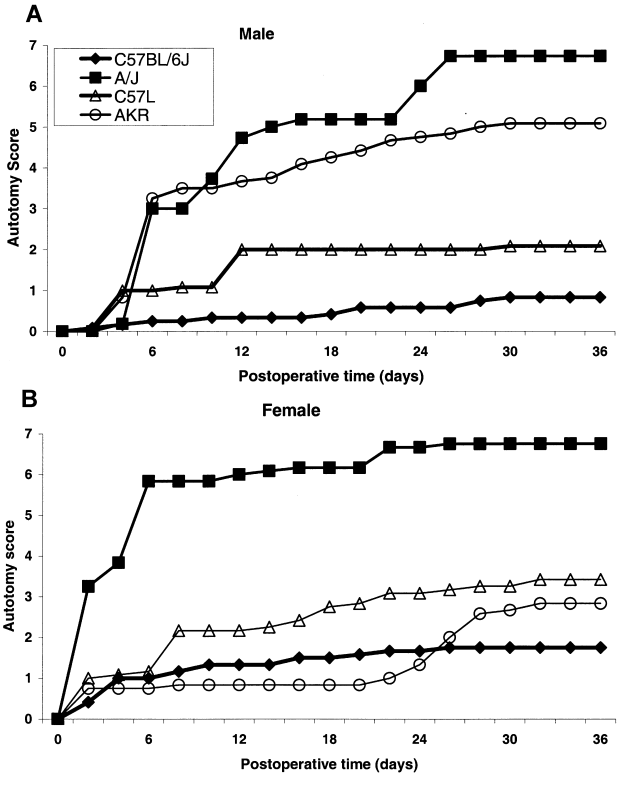

Fig. 1. Mean autotomy scores over 36 days following unilateral total hindpaw denervation for four inbred mouse lines that are progenitors of available RI sets. (A) males; (B) females. The greatest difference between lines was observed between A/J and C57BL/6J males at the termination of the protocol on day 36.

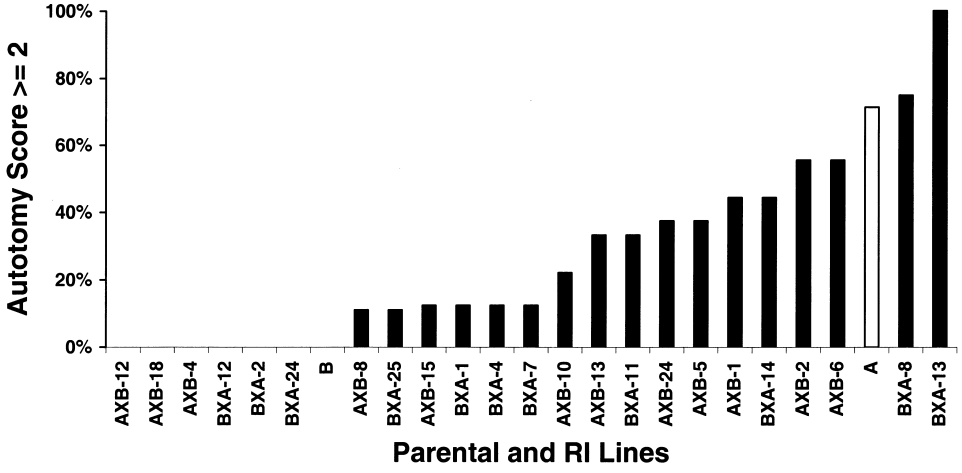

Fig. 2. Percentage of male mice with autotomy scores ≥2 for 23 RI lines of the AXB-BXA set and the A and B progenitor inbred lines used to develop this RI set. About half of the lines exhibit low frequency of autotomy, similar to the B progenitor line, and half exhibit high frequency of autotomy, similar to the A progenitor line.

mapping using AXB-BXA RI lines (Sampson et al., 1998). We further customized this database for use with the 23 RI lines included in our study, as described in the Methods, to produce our final database of 395 marker loci spanning the mouse genome. For each of these markers, we applied a statistical test that contrasts the frequency of animals with autotomy scores ≥2 in RI lines which inherited an A allele at the marker versus lines which inherited the B allele (Diehl and Erickson, 1997). If the marker is located near a gene which has a strong influence on autotomy, it is expected that a high percentage of animals in RI lines inheriting the A allele will display autotomy, while very few animals in RI lines inheriting the B allele will exhibit this behavior. A marker closely linked to a gene having a major effect on autotomy would thus be expected to produce a very large and statistically significant contrast between the two groups of lines sorted according to the marker's alleles.

Applying this test to our 395 markers spanning the mouse genome, we obtained a very strong contrast in frequency of autotomy between the two groups of RI lines which inherited the A versus the B progenitor allele for marker D15Mit28 ($\chi^2 = 18.0$, 1 d.f. $P = 0.00002$) as shown in Fig. 3. This marker is located on chromosome 15 at a position 43.7 centimorgans (cM) from the telomere. To assess statistical significance of this finding for a genome scan, the critical question is how likely a contrast of this magnitude is expected to occur by chance after adjusting for the multiple comparisons inherent in such an analysis (Lander and Kruglyak, 1995). To answer this question, we used a permutation strategy previously developed for binary response data evaluated in RI lines (Diehl and Erickson, 1997). We generated 2000 permuted data sets (randomly reassigning autotomy phenotypes among the RI lines) for our collection of 395 marker loci and analyzed the resulting 790 000 marker-autotomy phenotype combinations using the same categorical logistic regression method used for the actual data. We found only 238 markers out of 790 000 in the simulated data set had χ^2 statistics as great as that which we observed for D15Mit28 in the original study data. This frequency of 0.0003 is within the range between 0.00005 to 0.0007 that has been suggested as constituting significant evidence of linkage for genome wide mapping in the mouse (Lander and Kruglyak, 1995).

4. Discussion

The mouse genetic map for the region surrounding D15Mit28 is not precisely resolved, with data from these RI lines (Sampson et al., 1998) suggesting different marker orders than other sources (Blake et al., 2000). Furthermore, given the limited number of meioses and markers available in the AXB-BXA RI database, it is not possible to precisely

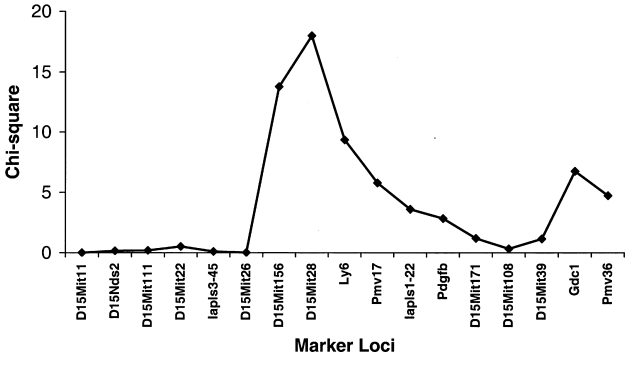

Fig. 3. Evidence of linkage to autotomy for marker loci on chromosome 15. The highest χ^2 statistic of 18.0 was obtained for the contrast of RI lines which inherited the A vs. B progenitor alleles at D15Mit28. Markers are displayed in the order best supported by their mapping in AXB and BXA RI lines (Sampson et al., 1998), with equal spacing between adjacent markers. Map positions in cM are not used in this illustration, because of inconsistencies of map order and distance for closely linked markers between various sources of mapping information (Blake et al., 2000).

determine the size of a candidate region implicated by our finding. Nevertheless, based on the reduced support for linkage provided by flanking markers we estimate that the autotomy gene lies within about 2–3 cM of D15Mit28. Several genes that play important roles in neural functions relevant to pain map within this region (Blake et al., 2000). *Pva,* (43.2 cM), encodes for the calcium-binding protein parvalbumin, implicated as a natural neuroprotectant against input excitotoxicity triggered by NMDA-mediated injury discharge emitted by damaged sensory fibers (Dubner, 1991; Seltzer et al., 1991). Since parvalbumin is mainly present in GABAergic neurons (Gao et al., 2000), their destruction may underlie the observed association of chronic disinhibition of afferent input with autotomy. Another candidate gene is *Bzrp* (43.3 cM), the peripheral benzodiazepine receptor, located on the mitochondrial membrane of astrocytes and microglia and whose expression is increased following neural injury (Raghavendra Rao et al., 2000) and inescapable stress (Lehmann et al., 1999). Chronic pain is caused by neural injury and causes in humans inescapable stress. In fact, diazepam, an anxiolytic agent and a ligand of these receptors, significantly suppresses autotomy in rats (Seltzer et al., 1989). The gene *Emo2* (43.3 cM) has not been cloned as yet but is mapped as a QTL associated with anxiety, fear and depression (Turri et al., 1999), behaviors commonly accompanying chronic pain in humans and highly correlated with autotomy in mice (Vatine et al., 2000). *Galr3* (46.3 cM) is the receptor for the galanin, a neuropeptide that affects autotomy (Ji et al., 1994) and nociceptive processing (Kerr et al., 2000) following nerve peripheral nerve injury. *Il2rb* (43.3 cM), the interleukin 2 receptor β chain, is implicated because knockout of other interleukins increases autotomy (Xu et al., 1997) and because IL-2 has been shown to inhibit nociceptive responses (Guo and Zhao, 2000). Additional candidate genes located in the vicinity of D15Mit28 (Blake et al., 2000) are *Cacng2,* (45.2 cM), the γ2 subunit of the voltage-dependent Ca^{2+} channel, *Kcnj4*(46.7cM), the K^{+} inwardly-rectifying channel and *Jrk* (42.8 cM), a gene recognized by a phenotype of clonic seizures and epileptic brain activity.

We noted that the 2 cM region around D15Mit28 appears to have a higher density of genes potentially relevant to autotomy than perhaps any other area of similar size in the mouse genome at its current level of resolution. It is possible that sequence differences between the A and B inbred lines in more than just one of the candidate genes in this region may contribute to the major differences in autotomy behavior observed between these lines. Because of this very high density of candidate genes in the region, an especially large number of genetic crosses, possibly complemented by transgenic studies, will be necessary to identify the specific gene or genes responsible for the autotomy locus mapped in our study. Identifying a gene having a major effect on autotomy may provide targets for the development of novel analgesics for neuropathic pain in humans.

Acknowledgements

We thank M. Devor for sharing data on autotomy levels of some progenitor lines used in the first part of this study. We acknowledge R. Oldakowski for assistance with SAS programming, R.P. Erickson. M. Iadarola and A. Notkins for suggestions for improving our manuscript, M. Iadarola and M. Kriete for animal protocol development and oversight, U. Goldberg for technical assistance and J. Riggs for manuscript preparation.

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